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Healing of osteoporotic bone defects by baculovirus-engineered bone marrow-derived MSCs expressing MicroRNA sponges

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ABSTRACT

Fractures associated with osteoporosis are a worldwide health problem. To augment osteoporotic bone healing, we aimed to develop a cell/gene therapy approach in combination with miRNA manipulation. We unraveled aberrant overexpression of miR-140* and miR-214 in the bone marrow-derived MSCs isolated from ovariectomized (OVX) rats (OVX-BMSCs). To suppress the miRNA levels, we constructed hybrid baculovirus vectors expressing miRNA sponges to antagonize miR-140* or miR-214. Engineering OVX-BMSCs with the hybrid vectors persistently attenuated the cellular miR-140*/miR-214 levels, which promoted the OVX-BMSCs osteogenesis and augmented the ability of OVX-BMSCs to repress osteoclast maturation *in vitro*. Notably, suppressing miR-214 exerted more potent osteoinductive effects. In the osteoporotic rat models with a critical-size bone defect at the femoral metaphysis, implanting the OVX-BMSCs ectopically expressing BMP2 failed to heal the defect, which underscored the difficulty to heal osteoporotic bone defects. Nonetheless, allotransplantation of the miR-214 sponges-expressing OVX-BMSCs healed the defect and ameliorated the bone quality (density, trabecular number, trabecular thickness and trabecular space) at 4 weeks post-implantation. Co-expressing BMP2 and miR-214 sponges in OVX-BMSCs further synergistically substantiated the healing. The baculovirus-engineered OVX-BMSCs that expressed miR-214 sponge, with or without BMP2 expression, thus paved a new avenue to the treatment of osteoporotic bone defects.

1. Introduction

Bone is a dynamic tissue that requires coordinated homeostasis of bone-forming osteoblasts and bone-resorbing osteoclasts to maintain constant bone mass and volume [1]. Osteoporosis arises from the dysregulation of bone turnover such that bone resorption exceeds bone formation, leading to reduction of bone mass and micro-architectural deterioration [1]. Osteoporosis affects as many as 75 million people in the US, Japan and Europe and ≈30–50% of women and ≈15–30% of men with osteoporosis have a fracture during their lifetime [2]. Since osteoporotic fractures are common (>9 million per year globally [1]), the lost productivity and healthcare expenditures have rendered osteoporosis a worldwide health problem [3,4]. To date, antiresorptive agents that inhibit osteoclast activities (e.g. bisphosphonates, calcitonin and estrogen) and anabolic agents (e.g. recombinant human parathyroid hormone) are available for osteoporosis treatment [4]. However, the repair of bone defects following fracture draws less attention.

MicroRNAs (miRNAs) are small non-coding RNAs that regulate gene expression by translation inhibition or mRNA degradation, through binding to the target mRNAs. MiRNAs modulate diverse biological processes, including osteoclastogenesis, osteogenesis, bone formation and can alter the bone phenotype (for review see Refs. [5–7]). For instance, miR-23a, miR-133, miR-335 and miR-3077-5p inhibit osteogenesis by directly interacting with the 3′ untranslated region (3′ UTR) of osteogenic transcription factor Runx2 [8]. Conversely, miR-21 targets the Fas ligand to suppress osteoclastic apoptosis, thereby supporting osteoclastogenesis [9]. However, little is known about the roles of miRNA in osteoporosis.

Mesenchymal stem cells (MSCs) play critical roles in bone formation as bone marrow-derived MSCs (BMSCs) are capable of

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differentiating into osteoblasts and then osteocytes. Baculovirus (BV) is a non-pathogenic insect virus and can transduce BMSCs and adipose-derived stem cells (ASCs) at efficiencies exceeding 90% [10,11]. We have shown that transduction of BMSCs or ASCs with BV carrying a transgene encoding osteoinductive bone morphogenetic protein 2 (BMP2) or miRNA (e.g. miR-148b) can enhance the osteogenic differentiation and promote the healing of critical-size bone defects after cell implantation into animals [12–19]. Normally, BV confers transient transgene expression [15] as BV genome exists as an episome and degrades with time [20], which eases the safety concern [21] but meanwhile restricts its applications. To extend the transgene expression, we developed a hybrid BV system, which comprises two BV vectors: one BV expressing Cre recombinase and the other substrate BV harbors the transgene cassette flanked by two loxP sites [22]. After co-transduction of ASCs with the Cre/loxP-based hybrid BV system, the Cre recombinase excises the loxP-flanking cassette off the substrate BV genome and catalyzes the formation of DNA minicircles, thereby prolonging the transgene (e.g. BMP2) expression [22].

Given the putative roles of miRNAs in osteoclastogenesis and osteogenesis and the need to heal osteoporotic fractures, we hypothesized that miRNA expression in osteoporotic BMSCs is dysregulated, hence impairing the ability of BMSCs to differentiate into osteoblasts for bone repair. Therefore, we created osteoporotic rat models by ovariectomy (OVX) and analyzed the miRNA expression profile in the OVX-BMSCs. We uncovered that miR-140* and miR-214 were overexpressed in OVX-BMSCs, and thus constructed Cre/loxP-based BV harboring miRNA sponges, in order to persistently knock down miR-140* or miR-214. Whether suppressing miR-140* or miR-214 was able to restore the osteogenesis of OVX-BMSCs and inhibit the osteoclast maturation was evaluated *in vitro*. We further assessed whether the BV-engineered, miRNA sponge-expressing OVX-BMSCs, with or without BMP2 co-expression, were able to heal critical-size bone defects in osteoporotic rats.

2. Materials and methods

2.1. Osteoporotic rat model, cells, BMSCs isolation and expansion

All animal experiments were performed in compliance with the Guide for the Care and Use of Laboratory Animals (Ministry of Science and Technology, Taiwan). To generate osteoporotic rat models, the Sprague–Dawley female rats (8 weeks old, BioLASCO, Taiwan) were subjected to bilateral ovariectomy (OVX) or sham operation (Sham) after anesthetization by intramuscular injection of Zoletil 50 (25 mg/kg body weight) and 2% Rompun® (0.15 ml/kg body weight). Under sterile conditions, a lumbar lateral incision was made between the caudal edge of the rib cage and the base of the tail. The ovary and part of the oviduct were removed with scissors, and the muscles and skin were sutured with resorbable suture. After ovariectomy, the rats were daily injected with methylprednisolone hemisuccinate (1 mg/kg body weight/day, Sigma) for four consecutive weeks [23].

BMSCs were harvested from the hind limb of OVX rats or Sham rats and the cells were flushed by 22G needle attached to a 10-ml syringe containing DMEM medium (Invitrogen). The cells were filtered through a 70-mm filter mesh to remove bone spicules and cell clumps, resuspended in DMEM medium containing 10% fetal bovine serum (FBS, Hyclone), 100 IU/ml penicillin and 100 IU/ml streptomycin, incubated at 37 °C with 5% CO_2 in a humidified chamber and were passaged 3–5 times for experiments. RAW 264.7 cells were cultured in RPMI-1640 medium (Invitrogen) supplemented with 10% FBS, 100 IU/ml penicillin and 100 IU/ml streptomycin.

2.2. Recombinant BV preparation and transduction

To construct donor plasmids (pBacECre, pBacLEW and pBacLEBW), the EF-1α promoter was PCR-amplified from pVITRO1-neo-mcs (Invivogen) and cloned into pBacCre, pBacLCW and pBacLCBW [24] using *XhoI*/*BamHI* to replace the CMV promoter because EF-1α promoter was stronger than the CMV promoter in rat BMSCs (data not shown). To generate pBac140S (encoding miR-140 sponge) and pBac214S (encoding miR-214 sponge), oligonucleotides encoding 10 tandem rno-miR-140* or hsa-miR-214 binding sites together with flanking 5′-*NotI* and 3′-*AflII* sites were chemically synthesized (Table S1) and cloned into pUC57 (Invitrogen) to yield pUC-miR140S and pUC-miR214S. The *NotI*-miR-140S-*AflII* (or *NotI*-miR-214S-*AflII*) fragment was *NotI*/*AflII* digested from pUC-miR140S (or pUC-miR214S) and subcloned into pd2EGFP-N1 (Clontech) to yield pd2EGFP-140S (or pd2EGFP-214S) (Fig. S1). Next, the *BamHI*-d2EGFP-miR-140S-*EcoRI* (or *BamHI*-d2EGFP-miR-214S-*EcoRI*) fragment was *BamHI*/*EcoRI* digested from pd2EGFP-140S (or pd2EGFP-214S) and subcloned into pBacLEW to yield pBac140S (or pBac214S) (Fig. S2).

All donor plasmids (pBacECre, pBacLEBW, pBac140S and pBac214S) were used to generate BV vectors (BacECre, BacLEBW, Bac140S and Bac214S) by using the Bac-To-Bac® system (Invitrogen) following the manufacturer's instructions. Virus titers were determined by end-point dilution method and are expressed as plaque forming units per milliliter (pfu/ml) [11].

BMSCs were transduced with BV vectors as described [11]. Briefly, rat BMSCs were seeded to 6-well plates (2×10^5 cells/well) or T-75 flasks (5×10^6 cells/flask), cultured overnight and washed twice with phosphate-buffered saline (PBS, pH 7.4) prior to transduction. Depending on the multiplicity of infection (MOI), a certain volume of virus supernatant was mixed with $NaHCO_3$-free DMEM at a volumetric ratio of 1:4 (total volume was 0.5 and 2 ml in 6-well plates and T-75 flasks, respectively) and added to the cells. For mock transduction, virus-free TNM-FH medium was mixed with $NaHCO_3$-free DMEM at a volumetric ratio of 1:4 and added to the cells. The cells were gently shaken on a rocking plate at room temperature for 6 h. After the transduction period, the virus mixture was replaced with the osteoinduction medium (DMEM containing 10% FBS, 100 IU/ml penicillin, 100 IU/ml streptomycin, 0.1 μM dexamethasone, 10 μM β-glycerol phosphate and 50 μM ascorbic acid 2-phosphate) containing 3 μM sodium butyrate (all from sigma). After 15 h of incubation at 37 °C, the medium was replaced by fresh osteoinduction medium.

2.3. Quantitative real-time reverse-transcription PCR (qRT-PCR)

Total RNA was isolated using the NucleoSpin RNA II kit (Machereye-Nagel) and reverse transcribed to cDNA using the Omniscript RT Kit (Qiagen). The osteogenic and osteoclastic genes were analyzed by qPCR using StepOnePlus Real-Time PCR Systems (Applied Biosystems) and gene-specific primers (Table S2). The mature miRNA was isolated using Trizol (Invitrogen) and analyzed using the TaqMan MicroRNA Assays kit (Applied Biosystems). The gene expression levels were normalized against that of U6 (for miRNAs) or *gapdh* (for osteogenic and osteoclastic genes) and referenced to that of the mock-transduced Sham-BMSCs (for miRNAs), mock-transduced OVX-BMSCs (for osteogenic genes) or Raw 264.7 co-cultured with mock-transduced Sham-BMSCs (for osteoclastic genes).

2.4. Alizarin red staining, calcium deposition assay and ELISA

The transduced OVX-BMSCs and mock-transduced BMSCs (OVX or Sham) were cultured with osteoinduction medium and stained